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Lunatic fringe causes expansion and increased neurogenesis of trunk neural tube and neural crest populations

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Abstract

Both neurons and glia of the PNS are derived from the neural crest. In this study, we have examined the potential function of lunatic fringe in neural tube and trunk neural crest development by gain-of-function analysis during early stages of nervous system formation. Normally lunatic fringe is expressed in three broad bands within the neural tube, and is most prominent in the dorsal neural tube containing neural crest precursors. Using retrovirally-mediated gene transfer, we find that excess lunatic fringe in the neural tube increases the numbers of neural crest cells in the migratory stream via an apparent increase in cell proliferation. In addition, lunatic fringe augments the numbers of neurons and upregulates Delta-1 expression. The results indicate that, by modulating Notch/Delta signaling, lunatic fringe not only increases cell division of neural crest precursors, but also increases the numbers of neurons in the trunk neural crest.

Keywords

neural crest; lunatic fringe; notch; cell division

INTRODUCTION

The vertebrate nervous system arises during neurulation as the ectoderm becomes partitioned into neural and non-neural domains. The neural ectoderm thickens to become the neural plate, which invaginates to form the neural tube. Presumptive neural crest cells initially lie in the neural folds at the juncture between the neural and non-neural ectoderm and then within the dorsal neural tube after its closure. These cells then undergo an epithelial to mesenchymal conversion and subsequently emigrate from the neural tube as neural crest cells. After leaving the neural tube, neural crest cells migrate to characteristic destinations in the embryo, forming cell types as diverse as smooth muscle and cartilage to sensory neurons and glia of the PNS. Previously, we showed that lunatic fringe enhances proliferation of cranial neural crest cells (Nellemann *et al.*, 2001), but its consequences on cell fate decisions of neural crest cells and its role in the trunk were not examined.

Notch signaling has been shown to be a key player in important developmental events including cell-fate specification, induction and lateral inhibition (Louvi and Artavanis-Tsakonas, 2006). The Notch receptor participates in a broad spectrum of developmental processes and at a variety of times by interacting with its ligands Delta, Jagged and Serrate (Irvine, 1999; Wang and Barres, 2000). The fringe family of extracellular secreted proteins is a modulator of Notch–

Delta activity. In *Drosophila*, fringe encodes a secreted glycosyl-transferase that plays an important role in boundary-specific signaling during pattern formation (Johnston *et al.*, 1997; Barrantes *et al.*, 1999). It localizes the signaling activity of Notch by Delta to presumptive boundary regions such as the *Drosophila* wing margin while blocking Notch response to Serrate (Panin and Irvine, 1998; Ju *et al.*, 2000; Moloney *et al.*, 2000), and is known to influence Notch signaling in a cell autonomous manner by forming complexes with Notch before secretion (Ju *et al.*, 2000; Sakamoto *et al.*, 2002). In this way, fringe modulates the ligand preference of Notch for Delta.

In vertebrates, there are three identified homologues of fringe (lunatic, manic and radical) (Johnston *et al.*, 1997). Lunatic fringe is expressed in an oscillatory pattern in the presomitic mesoderm (McGrew *et al.*, 1998) and mutations in this gene result in severe somite segmentation defects caused by periodic inhibition of Notch (Eph Nomenclature Committee, 1997; Evrard *et al.*, 1998; Dale *et al.*, 2003). Both lunatic fringe and radical fringe are expressed highly in the developing nervous system of vertebrate embryos (Johnston *et al.*, 1997), although little is known about their function therein. Moreover, lunatic and radical fringe are expressed between brain boundaries during development in the mouse and zebrafish, and its expression has been shown to be crucial for proper boundary determination (Larsen *et al.*, 2001; Zeltser *et al.*, 2001; Cheng *et al.*, 2004). Katsube and co-workers have shown that ectopic expression of lunatic fringe downregulates serrate in the neural tube, but they did not examine effects on either neural crest formation or neurogenesis (Sakamoto *et al.*, 1998).

Here, we examine the effects of excess expression of chick lunatic fringe on trunk neural crest cells and explore its possible function using retrovirally-mediated gene transfer during the early stages of nervous system formation. Normally lunatic fringe is expressed in the neural tube from which neural crest cells emerge. When expressed ectopically in the developing nervous system, lunatic fringe increased the numbers and density of migrating neural crest and promoted neuronal differentiation, and upregulated expression of neuronal markers. Furthermore, its ectopic expression increased *Delta-1* transcripts in the neural tube. Our previous finding showed that over-expression of lunatic fringe in the neural tube and neural crest resulted in a profound increase in cell number at early migratory stages (Nellemann *et al.*, 2001). Here we propose a wider role for lunatic fringe: we suggest that lunatic fringe not only enhances neural crest cell division but, importantly, it can increase neuroblast cell division and neurogenesis from both neuroblast and neural crest cells.

OBJECTIVE

The goal of this study was look at the potential function of lunatic fringe in neural tube neuroblasts and trunk neural crest development by gain-of-function analysis during early stages of nervous system formation.

METHODS

Retroviral infection of chick embryos with RCAS lunatic fringe retrovirus

Cloning of lunatic fringe and viral production was performed as described previously (Hughes *et al.*, 1987). Control viruses consisted of empty vector. Viruses were produced according to published procedures (Morgan and Fekete, 1996). Primary cultures of chicken embryo fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum, 2% chicken serum and 1% penicillin and streptomycin. Dishes (80% confluent) were transfected with 6 µg of viral DNA using Lipofectamine (GIBCO) following the manufacturer's instructions. Virus was propagated in transfected chicken embryo fibroblasts passaged for a week to ensure virus spread throughout the culture. Confluent cells were given a minimum volume of low-serum media (2% FCS, 0.2% chick serum in DMEM) and the media

from 24-hour cell cultures was collected, centrifuged for 15 min at 3000 rpm to remove cells and debris, and then ultracentrifuged for 3 hours at 30 000 rpm. The pellet with viral particles (titer $\sim 10^8$) was solubilized in ~ 100 μ l of DMEM and kept in liquid nitrogen.

Fertilized eggs were incubated at 37°C for ~ 28 hours. Eggs were windowed and visualized by a sub-blastodermal injection of India Ink [diluted 1:10 in phosphate buffered saline (PBS)]. Retrovirus plus 0.8 μ g l⁻¹ of polybrene was backfilled into a micropipette and injected in the closing neural tube. The eggs were closed with Scotch tape and reincubated for an additional 24 hours.

Embryo fixation and *in situ* hybridization

Embryos were removed from the eggs and stripped of the membranes. They were fixed in 4% paraformaldehyde overnight before being stored in 0.1 M PBS.

Patterns of gene expression were determined by whole-mount *in situ* hybridization using DIG-labeled RNA antisense probes as described by Henrique *et al.* (Henrique *et al.*, 1995). The 800 bp c-Notch-1 and 550bp c-Delta-1 probes used are described in Myat *et al.* (Myat *et al.*, 1996).

Analysis of cell division with BrdU labeling

Embryos were incubated for 20–24 hours after retroviral infection before being opened and given 5.1×10^{-2} M BrdU (Sigma) dissolved in double distilled H₂O. The BrdU solution was placed over the heart, a location that leads to import of the BrdU in head regions. The embryos were incubated with the BrdU solution for 1 hour and harvested.

Cryostat sectioning and immunohistochemistry

For cryostat sectioning, fixed embryos were placed in 5% followed by 15% sucrose overnight at 4°C, embedded in 15% sucrose/7.5% gelatin (Sigma) (Sechrist *et al.*, 1995), rapidly frozen in liquid nitrogen and serially sectioned at a thickness of 10–20 μ m on a cryostat (Zeiss Micron, Heidelberg). Sections were mounted on subbed slides.

After retroviral infection, sections were stained with HNK-1 antibody to recognize neural crest cells or p27 to recognize viral proteins. Approximately 20 μ l of antibody solution was applied to each section and incubated overnight in a humidified chamber either at 4°C or 25°C. After incubation with primary antibodies, sections were washed in PBS and incubated for 1–1.5 hours with either FITC or Alexa Red™ conjugated antibodies against mouse IgM (Collaborative Research) for recognizing the HNK-1 antibody or anti-rabbit IgG (Molecular Probes). Sections were washed in PBS and cover-slipped with permafluor (Immunon). No significant fluorescent signal was detectable with the secondary antibodies alone.

After BrdU treatment, sections were treated with 2N HCl for 20 min, washed in PBS and incubated at 4°C overnight with 1:30 mouse IgG against BrdU and 1:500 anti-p27. The sections were washed in PBS extensively, treated at room temperature for 3 hours with 1:300 Hi-FITC-labeled goat-anti-mouse IgG and TRITC-labeled goat-anti-rabbit IgG before being washed in PBS, mounted with fluorochrome G and visualized using a conventional fluorescence microscope.

In vitro neural crest culture

Chicken neural tubes from HH14–16 were dissociated in 1.5 mg ml⁻¹ of Dispase and washed in Leibovitz-15 media. The neural tubes were cut in small pieces (size of 2–3 somites) and pipetted in the center of wells coated with fibronectin (10 μ g ml⁻¹). Neural tubes were cultured in DMEM and 10% fetal bovine serum (FBS) and 100 mg ml⁻¹ and 100 U of penicillin and

streptomycin respectively for 18 hours after which they were fixed in 4% paraformaldehyde for 30 min and subsequently blocked for 30 min with PBS, 1% Triton-X100, 10% FBS. Primary antibody was either HNK-1 or TuJ1 for visualizing neural crest cells and neurons, respectively, followed by an anti-mouse IgM-Alexa 488 secondary (Molecular Probes). At the end, slides were incubated with DAPI in PBS to visualize cell nuclei.

Electroporation of chick embryos with RCAS lunatic fringe retrovirus

A 3 mg ml⁻¹ solution of DNA was pressure-injected under the vitelline membrane and into the neural tube, open neural plate, using pulses of compressed air from a picospritzer (General Valve). Two, platinum, L-shaped wires were placed 2 mm apart on either side of the neural tube. Two 50 msec electrical square wave pulses of 25 mV were applied to the embryo using a pulse generator. Embryos were moistened with Ringer's solution, sealed with tape and reincubated. After 1–2 hours, embryos were dissected, their neural tubes dissociated with disperse and cultured on fibronectin-coated glass slides. Infected neural crest cells were visualized with HNK-1 and p27 simultaneously, followed by an anti-mouse IgM-Alexa 488 and anti-rabbit IgG-Alexa 594 specific secondary antibodies (Molecular Probes).

RESULTS

Expression pattern of lunatic fringe in the trunk region

In the trunk region of the chick embryo, lunatic fringe was expressed robustly in the developing nervous system. There was abundant staining in the neural tube and open neural plate along the rostral-caudal extent of the neural axis. After neural tube closure, transverse sections reveal that expression was most prominent in a broad band in the dorsal neural tube; in addition, there was a less prominent stripe in the intermediate portion of the neural tube and a third broad band in the ventral half of the neural tube (Fig. 1A). Interestingly, lunatic fringe transcripts are apparently absent from the most lateral aspects of the neural tube, where cells are undergoing active differentiation. Faint expression was detected in migrating neural crest cells within the sclerotome (arrowhead in Fig. 1B). As previously described, a dynamic pattern of expression is apparent in the paraxial mesoderm as the somites are generated (Jouve *et al.*, 2002).

Effects of over-expression of lunatic fringe on neural crest cells

The pattern of lunatic fringe expression in the developing nervous system is intriguing because it overlaps partially with that of Notch-1 and Delta-1. To explore the role of lunatic fringe in an *in vivo* developing system, we over-expressed transcripts via retroviral infection of the neural tube of embryos with approximately 12–28 somites (stages HH11–16, *n* = 96) according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951) by injecting the virus inside the neural tubes. Injection of retrovirus into this region resulted in infection of the neural crest, neural tube and sometimes the surface ectoderm (Fekete and Cepko, 1993). Embryos were subsequently fixed 1–2 days after viral infection (HH17–21). Infection of cells by retrovirus was monitored by staining for the viral protein p27. The effects on the distribution of neural crest cells were assayed by sectioning the embryos and staining with the HNK-1 antibody, which marks migrating neural crest cells.

In embryos examined 1 day after infection, we noted a marked increase in the numbers of migrating neural crest cells in lunatic fringe-infected compared with control embryos (Fig. 2A–D). Immunostaining with the p27 marker for RCAS virus (red staining in Fig. 2A–F) was used to determine the levels of viral infection in the embryos. We observed significant presence of viral particles in neural tube neuroblasts and neural crest cells (observable as yellow staining for double labeling of p27 and HNK1 in Fig. 2A–D). Although there was very little p27 staining in the surrounding mesenchyme after 24 hours after infection, we observed that after 48 hours of infection, the surrounding mesenchyme was positive for p27, probably because the viral

particles were able to infect surrounding tissues by this time (24 control and 20 experimental embryos were counted). Despite the increase in the numbers of migrating neural crest cells after ectopic expression of lunatic fringe, no differences were noted in the migratory pathways followed by these cells. Rather, they appeared to occupy a somewhat larger proportion of the mesenchyme than in control-infected embryos of a similar stage (arrows in Fig. 2A–D). Furthermore, the lunatic fringe-infected neural crest streams had significantly more cells adjacent to the neural tube in the region where the dorsal root ganglia (DRG) will condense (~25% increase) compared with control infected embryos (control, 53 ± 3 ; lunatic fringe, 65 ± 5 nuclei per 20 μm section through the forming DRG, $P < 0.03$, Student's *t*-test). Thus, the neural crest stream appeared to have a higher cell packing density after lunatic fringe infection.

Based on the increase in neural crest cells at day 1 post-infection, we expected the DRG to be larger at day 2. However, 2-days post-infection (~HH20), when DRG are in the process of condensing, the size of the DRG was comparable in lunatic fringe and control embryos (Fig. 2E–F). Because the increase at day 1 did not result in increased neurons at day 2, it is likely that some of these new cells did not survive. One interesting difference within the ganglia was that the distribution of HNK-1-positive cells appeared higher in the periphery of the ganglia than in the interior in experimental but not in control embryos (Fig. 2E–F). Because proliferating precursors tend to localize in this capsule region, these results indicate that there might be more differentiating neurons in the center of the DRG of lunatic fringe-infected embryos.

Lunatic fringe over-expression enhances neurogenesis in both the PNS and CNS

The apparent increase in the numbers of trunk migrating neural crest cells indicated that lunatic fringe might alter differentiation, increase cell proliferation and/or reduce cell death in this cell population. To look for specific effects on early cell differentiation, embryos were stained with antibodies to neuron-specific markers, either β -tubulin or neurofilament, 1–2 days after infection. In control embryos infected at HH9 and examined at HH14, we observed very few differentiating neurons in the neural tube, as has been shown in the past, and most were localized primarily around its periphery (Fig. 3A). After infection with lunatic fringe, we noticed an increase of ~2 fold in the number of neurons in the neural tubes. There was also an increase in β -tubulin staining in the axonal processes across the width of the neural tube (Fig. 3B). We did not observe β -tubulin-positive cells in the region covered by neural crest because these cells are just initiating migration. In embryos immunostained with neurofilament antibodies 2 days post-infection, there was an increase in the numbers of neurofilament-positive cells in the forming DRG of lunatic fringe-infected embryos (Fig. 3C–D). Similar to β -tubulin and neurofilament, we noted an increase in neurogenin-1 and -2 (data not shown), which are expressed in developing neural crest-derived sensory neurons (Ma *et al.*, 1996; Ma *et al.*, 1998). Increased numbers of neurons were also observed in other regions of the nervous system; for example, both the retina and cortex of lunatic fringe-infected compared with control-infected embryos appeared to have augmented neurogenesis as determined by a thicker layer of neurofilament-positive cells (data not shown). In addition to affecting the nervous system, we noted an increase in the number of red blood cells in both the neural tube and dorsal aorta (arrowhead in Fig. 2F and data not shown).

Because it is difficult to accurately quantitate changes in cell proliferation and neurogenesis *in vivo* in embryos whose neuroblasts are all undergoing extensive cell division, we next carried out parallel experiments in tissue culture either by infecting neural tubes (HH14–16) with either lunatic fringe or control retrovirus or by electroporating viral DNA into neural tubes (HH14–16) and then explanting these on fibronectin to culture the migrating neural crest cells that come out of the tube as a halo of cells. As done before for embryo sections, we monitored the levels of viral infection in our cultures by p27 staining (Fig. 4A,C). We studied cultures that

showed extensive infection by RCAS virus because not all the cells in the culture are p27-positive (arrowheads in Fig. 4), as is expected in such short-term cultures (18hrs) where the virus is dispersed in the culture media. Retroviral infection of whole embryos is more efficient than of cultured neural tubes presumably because the viral particles can easily infect neighboring cells.

We observed that, similar to the *in vivo* results, excess lunatic fringe enhanced the early differentiation of neural crest into neurons and that neural tube cells infected with lunatic fringe had more neuronal processes than control cultures (arrows in Fig. 4B,D). We also noted an increase in the numbers of neurofilament-positive cells in lunatic fringe-infected neural tube cultures than in controls. These data indicate that, lunatic fringe enhances neurogenesis of neural tube neuroblasts *in vitro* as well as *in vivo*.

Cell division after ectopic expression of lunatic fringe

The increased percentage of neural crest cells within the forming DRG and the increased numbers of neurofilament-positive cells in lunatic fringe expressing embryos might be caused by lunatic fringe-induced cell proliferation (Nellemann *et al.*, 2001). To test this possibility, we quantitated the amount of cell division by examining the incorporation of BrdU both *in vivo* and *in vitro* in neural tube cultures post-infection with either control or lunatic fringe retrovirus to determine the proportion of dividing cells. In the whole embryo, we counted the numbers of BrdU/p27 in forming DRG that were uniformly infected with either lunatic fringe or control retrovirus. We observed a six-fold increase in BrdU incorporation in neural crest settling to form the DRG after infection with lunatic fringe retrovirus (Table 1).

In neural crest cultures, we also observed an increase in cell proliferation (Fig. 5A–D) although this was slightly less (~2 fold) than that seen in embryos. Cultures were labeled for 30 min with BrdU before fixation and p27 used as a marker for successful viral infection. The number of cells positive for both BrdU and p27 was compared with the total number of infected, p27-positive cells in the neural crest population. We found that an average of 31% of cells in control-infected neural crest cultures compared to 46% of cells infected with lunatic fringe incorporated BrdU and therefore were dividing (Table I). Thus, there is a 15% increase in the total number of neural crest cells that divide after lunatic fringe over-expression, which corresponds to a 1.5-fold increase in cell division. This increase in cell division was confirmed by staining lunatic fringe-infected neural crest cultures with anti-phospho-histone antibody, which detects cells in mitosis (Fig. 5E–F).

This difference between BrdU results obtained *in vivo* and *in vitro* after infection with the identical virus probably results from a lack of appropriate trophic factors in the culture media, which prevents neuroblasts and neural crest cells in culture from dividing to comparable levels as in a developing embryo. This was confirmed by a significant reduction in the number of cells that express neural markers after changing the media from one enriched with chicken embryo extract and FCS to defined medium (which lacks the trophic factors associated with serum and embryo extract, data not shown).

In addition to an increase in the number of neural crest cells at later stages, there was an increase in the size of the neural tube relative to head size in infected embryos, as exemplified by folding of the neural tube in many embryos. This appeared to be caused by a dramatic increase in neuroblast division in embryos 2 days after infection. This was particularly evident at cranial levels, where severe abnormalities such as overgrowth and abnormal shape of the midbrain were apparent in ~15% of lunatic fringe-infected embryos (data not shown).

Effects of lunatic fringe over-expression on Notch-1 and Delta-1

Neural crest cells continue to emerge from the dorsal neural tube for ~36 hours after their initial migration. Because lunatic fringe has been proposed to potentiate Notch-Delta signaling (Panin *et al.*, 1997; Moloney *et al.*, 2000), we tested whether lunatic fringe over-expression altered the levels or distribution patterns of members of the Notch signaling pathway to better understand the mechanisms by which lunatic fringe might regulate trunk neural crest production. In particular, we examined the effects of excess lunatic fringe on Notch-1 and its ligand Delta-1. The possible involvement of Serrate ligands was excluded from these studies because these ligands are not expressed in either the dorsal neural tube or neural crest cells (Hayashi *et al.*, 1996; Myat *et al.*, 1996). Embryos were infected with retrovirus encoding chick lunatic fringe, allowed to develop for 1–2 days and then examined by *in situ* hybridization for the expression patterns of Notch-1 and Delta-1. Most embryos were analyzed at stage 18–21 of development.

Normally, Delta-1 is expressed in a subpopulation of neural tube cells (Bettenhausen *et al.*, 1995), specifically in cells that have just exited the cell cycle to begin differentiation into neurons, and in the pre-somitic mesoderm and in the intermediated neural plate from which neural crest originates (Chitnis *et al.*, 1995). In embryos infected with lunatic fringe retrovirus, there was an upregulation of Delta-1-expressing cells in the pre-somitic mesoderm, as shown before (Dale *et al.*, 2003). There was also an increase in the intermediate neural plate that will give rise to neural crest precursors (arrowheads in Fig. 6A,B). In addition, the number of individual neural tube cells expressing Delta-1 appeared to increase, as seen in transverse sections through embryos at HH13. The average number of Delta-1-positive cells per embryo in control-infected embryos was 9.6 ± 2.6 compared with 15.3 ± 0.8 in lunatic fringe-infected embryos (a 60% increase). These results indicate that over-expression of lunatic fringe leads to increased number of cells that express Delta-1.

Conversely, we observed a reduction in the expression of Notch-1 mRNA after lunatic fringe infection in the intermediate neural plate (arrows in Fig. 6E,F). In neural tubes, Notch-1 mRNA is distributed throughout the rostro-caudal length of the neural tube in a relatively uniform pattern (red arrowhead in Fig. 6G). After over-expression of lunatic fringe (red arrowhead in Fig. 6H), the levels of Notch expression appeared to concentrate slightly more in the ventricular side of the neural tube and we observed reduced expression in the ventral neural tube. This region corresponds to the location of proliferating neuroblasts, indicating that cells in this region of the neural tube might have left the cell cycle earlier than in control-infected embryos. As we observed previously at cranial levels (Nellemann *et al.*, 2001), these distinctions were not significantly different from controls.

CONCLUSIONS

- Infection with lunatic fringe increases the number of neural crest cells in the migratory stream.
- Excess lunatic fringe in the neural tube stimulates the proliferation of neural crest cells and neural tube neuroblasts.
- Lunatic fringe infection induces early differentiation of neural tube neuroblasts and neural crest-derived neurons *in vivo* and *in vitro*.
- Lunatic fringe increases the number of Delta-1-expressing cells and reduces Notch expression.

DISCUSSION

The molecular components of Notch signaling pathways are highly conserved across the animal kingdom. Although vertebrate fringe genes are prevalent in the developing nervous system, little is known about their function. Here, we have examined the possible role of chick lunatic fringe during early neural tube and neural crest development. Lunatic fringe is expressed in bands within the trunk neural tube, including the dorsal-most region from which neural crest cells emerge. Single cell-lineage analysis has revealed that cells in this dorsal neural tube domain can form both neural crest and neural tube cells, indicating that these are multipotent precursors (Bronner-Fraser and Fraser, 1988). This domain also overlaps with expression of both Notch and Delta.

Using retrovirally mediated gene transfer we over-expressed lunatic fringe in the neural tube and neural crest in chick embryos to examine the effects of lunatic fringe on the early development of the nervous system. Lunatic fringe-infected embryos had a significant increase in the number of migrating neural crest cells, and in the amount of neural crest proliferation and in the number neurofilament-positive cells. This study corroborates our previous results with cranial neural crest (Nellemann *et al.*, 2001) and expands the role of lunatic fringe as a modulator of the Notch pathway and of expanding neuroblast and neural crest cell populations. We show that not only is lunatic fringe capable of increasing neural cell proliferation, but it also promotes neurogenesis.

Notch activation has been implicated in proliferation and maintenance of neural stem cells (de la Pompa *et al.*, 1997; Varnum-Finney *et al.*, 1998; Walker *et al.*, 1999; Savill and Sherratt, 2003). Our present results are consistent with these new findings. We find that 24 hours after infection with lunatic fringe, there are more proliferating cells in the condensing DRG compared with control. These results confirm the importance of Notch in neural stem cell-proliferation, because the neural crest itself has stem cell properties (Bronner-Fraser and Stern, 1991; Morrison *et al.*, 2000). Our findings indicate that Notch can influence neural crest stem cell proliferation *in vivo* (Hitoshi *et al.*, 2002), as well as causing them to exit their cell cycle and differentiate *in vitro* sooner than in control cultures. Previous studies have shown that activation of Notch in retinal progenitors inhibits differentiation and causes abnormal growth (Furukawa *et al.*, 2000). Furthermore, Harris and co-workers (Ohnuma *et al.*, 2002) showed that in *Xenopus*, early cell-cycle exit enhances determination of retinal fate in a manner similar to that seen in our study with excess lunatic fringe, where proliferating neural crest cells seem to differentiate sooner into neurons.

Although Notch signaling is generally thought to affect cell-lineage decisions, other data are consistent with this signaling pathway influencing cell proliferation. In the developing vertebrate nervous system, the Notch signaling pathway has been proposed to maintain a population of dividing, uncommitted precursor cells in the ventricular zone (Chitnis *et al.*, 1995; Lewis, 1996). In this area of the neural tube, cells proliferate extensively and give rise to post-mitotic neurons; after the period of neurogenesis, they form glial cells, with the former preceding the latter by several days. Initially, the number of neurons outnumbers the glia during prenatal development. One interesting possibility is that signaling between the ventricular zone and adjacent regions determines the proportion of cells that becomes post-mitotic versus those that continue to divide. In the retina, activation of Notch clearly keeps cells cycling (Bao and Cepko, 1997). The present data indicate that one potential role for lunatic fringe is to keep cells in a hyper-proliferative state. By enhancing Notch signaling, lunatic fringe might place the normally highly proliferative neural crest population into a hyper-proliferative state. This is likely to be true for neural crest cells as well as other cell types. For example, the size of the neural tube appeared to increase significantly in older experimental embryos, which showed

dramatic head malformations and excess blood cells (Supplementary Fig. 1). Both these phenotypes might result from excessive proliferation.

The present finding of increased neurogenesis induced by lunatic fringe complements those previously observed in the Delta-1-null mice, which have a neural crest phenotype (Hrabe de Angelis *et al.*, 1997). More importantly, lunatic fringe-null mice have severe reduction in sensory ganglia size, which indicates fewer neurons (Evrard *et al.*, 1998; Zhang and Gridley, 1998). During formation of the DRG in these mutant mice we noted that the initial rate of neurogenesis and gliogenesis was significantly impaired (de Bellard *et al.*, 2002). Together with the present results we hypothesize that, in addition to modulating Notch/Delta signaling, lunatic fringe might play a role in the Notch/Delta interactions that are important for maintaining the pool of progenitors from which neurons and glia derive, thus accounting for the observed increase in neurogenesis. The dramatic difference in the number of neurons present in our cultures after lunatic fringe infection depended on media conditions that favor neuronal survival (Supplementary Fig. 2). Lack of survival between day 1 and 2 might result from lack of trophic factors at this early stage in the embryo. Unlike differentiated neurons, neural crest cells do not need neurotrophins. In our experiments by day 1 we observed early neurogenesis, so these new cells require factors before they are available in the developing embryo. This hypothesis is supported by our results showing that when we add embryo extract to cultures, the day 2 group of cells survives. In other words, the survival of our lunatic fringe-infected neural tubes cultures depends on the addition of embryo extract whereas control cultures do not. This indicates that neurogenesis occurs earlier in our lunatic fringe infected cultures. Although others have suggested that Notch might inhibit differentiation while either allowing or stimulating cells to proliferate (Varnum-Finney *et al.*, 1998; Louvi and Artavanis-Tsakonas, 2006), our results support a role of Notch in proliferation, early differentiation and/or early exit from the cell cycle in order to differentiate (Molofsky *et al.*, 2004).

In addition to influencing neural crest proliferation, later activation of Notch through Delta in the neural crest lineage has been shown to influence cell-fate decisions in the neural crest: some migrating trunk neural crest cells express Notch-1 and its activation by Delta-1 promotes gliogenesis at the expense of neurogenesis (Morrison *et al.*, 2000; Wakamatsu *et al.*, 2000). However, in the chick, the first glial cells appear around embryonic day 7, which is well after the conclusion of the experiments conducted here. Therefore, we were unable to examine whether lunatic fringe simultaneously enhances neurogenesis and decreases gliogenesis.

In conclusion, our data indicate a previously unknown role for vertebrate lunatic fringe as a modulator of both proliferation of neural crest cells and neuroblast differentiation. Over-expression of lunatic fringe in the developing nervous system results in a large increase in cell number via increased proliferation of the neural tube and neural crest cells. Ectopic expression of lunatic fringe upregulated Delta-1 transcripts in the dorsal neural tube cells from which neural crest cells emigrate. These studies indicate an important function for lunatic fringe in the modulation of Notch signaling and controlling the number of precursors in the developing nervous system.

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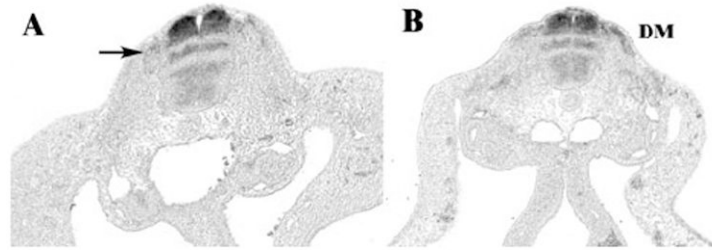


Fig. 1. Pattern of lunatic fringe expression in a stage 20 chick embryo at the level of the forelimb (A) and midtrunk (B)

Lunatic fringe is expressed robustly in the neural tube. In transverse section, three bands of staining are apparent; a broad band encompasses the dorsal neural tube, there is a narrow intermediate stripe, and a broad, less-distinct band in the ventromedial half of the neural tube. Expression was absent from the marginal zone. Lunatic fringe expression was also observed in the dermomyotome (DM) and at low levels in the migrating neural crest within the sclerotome (arrow).

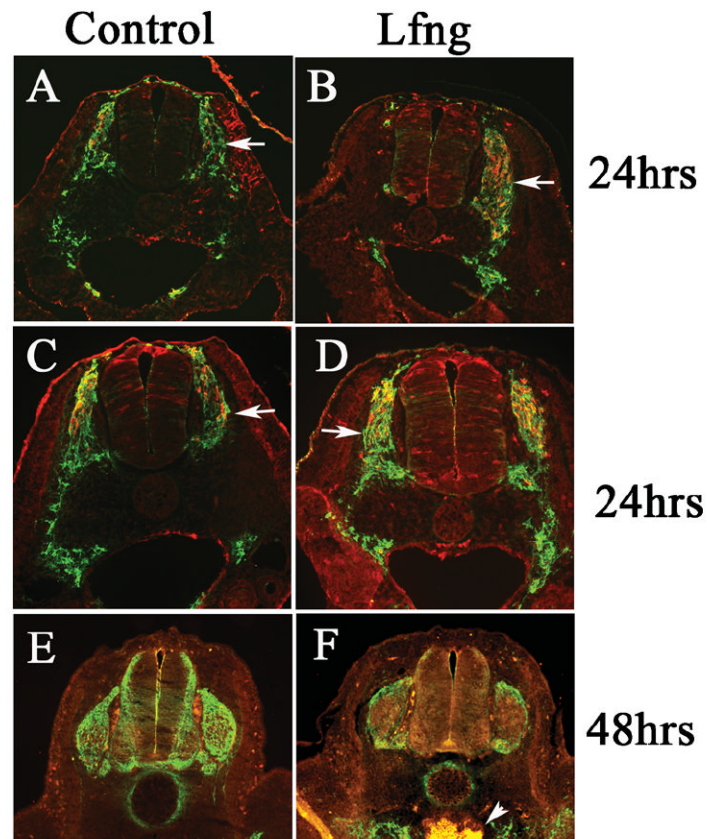


Fig. 2. Lunatic fringe infection increases the neural crest population

Neural tubes from HH11–12 chicken embryos were infected with either control or lunatic fringe (Lfng) virus, then allowed to develop for either 24 hours (A–D) or 48 hours (E–F). Embryos were fixed, sectioned and stained with the neural crest marker HNK1 (green) or the viral marker p27 (red) to visualize the infected cells. At 1 day post-infection, lunatic fringe-infected embryos (B,D) had more migrating neural crest cells than stage-matched control-infected embryos (A,C; white arrows). Analysis of cell density revealed a large increase in the number of migrating neural crest cells. At 2 days post-infection, the DRG in lunatic fringe-infected embryos (F) have more HNK1 immunostaining than controls (E) and this appeared to label the capsule rather than the whole ganglion.

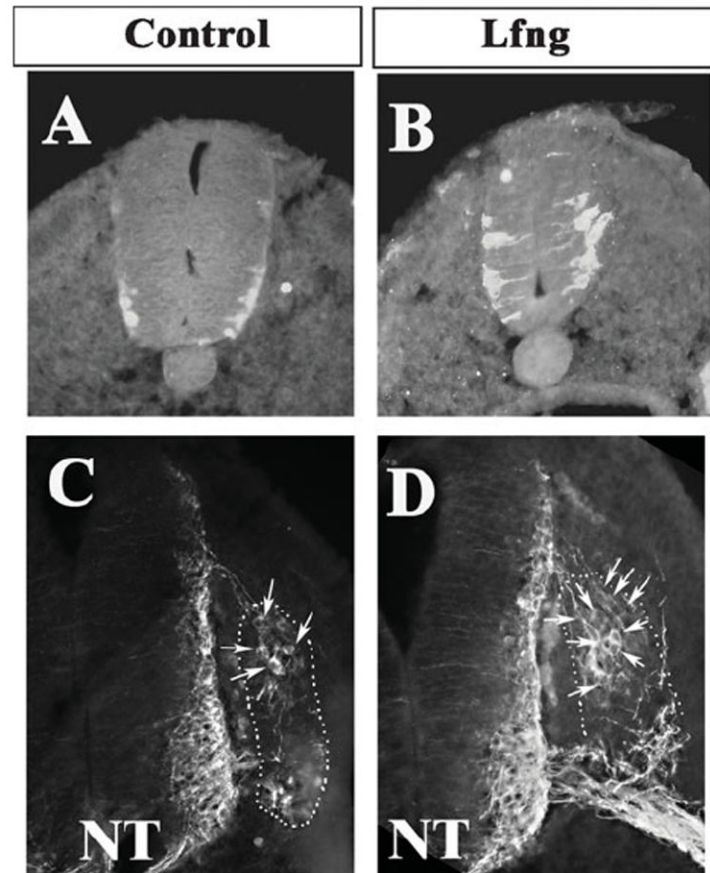


Fig. 3. Lunatic fringe infection induces differentiation of neural tube neuroblasts and neural crest-derived neurons

Neural tubes from HH9 (A,B) or HH15–17 (C,D) chicken embryos were infected with control or lunatic fringe virus and allowed to develop for 24–48 hours. Embryos were fixed, sectioned and stained with the neural marker anti- β tubulin (A,B) or neurofilament (C,D). (A,B) At 1 day post-infection, lunatic fringe-infected neural tubes had approximately 2-fold more neurons, indicative of a terminally differentiated cell. In addition, neurofilament-immunoreactive cells appeared to span the width of the neuroepithelium in lunatic fringe-infected embryos but appeared mostly in the periphery in control embryos (C,D). In older embryos, there appeared to be more neurofilament-positive neurons in the forming DRG. Arrows indicate neurofilament-positive neurons and dotted line indicates the outline of the condensing ganglion. NT, neural tube.

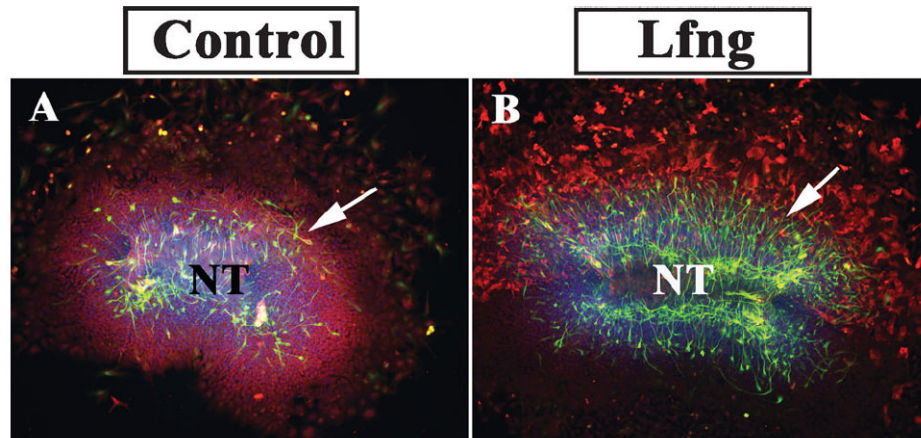


Fig. 4. Lunatic fringe increases cell differentiation *in vitro*

Neural tubes (NTs) were electroporated with either control (A) or lunatic fringe (Lfng) (B) retrovirus, dissected and plated onto fibronectin-coated slides, allowing the neural crest cells to migrate out. Cultures were immunostained with β -tubulin neuronal-specific marker (green) and for viral p27 protein (red). At 1 day post-infection there were many more neurites after infection with lunatic fringe than in similarly treated control neural tubes (arrows).

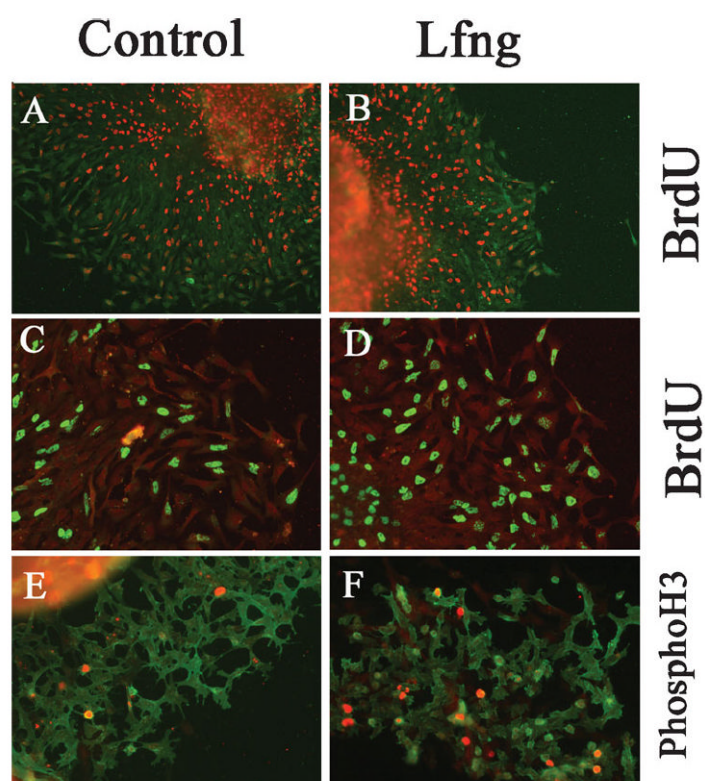


Fig. 5. Lunatic fringe increases cell division

Neural tubes from HH13–15 chicken embryos were infected with either control (**A,C and E**) or lunatic fringe (**B,D and F**) retrovirus and allowed to develop in culture for 24 hours allowing neural crest cells to come out. BrdU (10 μ M) was added for 30 min and cultures were fixed and stained for the p27 viral marker and either BrdU labeling or for phospho-histone (phosphoH3). Lunatic fringe-infected crest cells incorporated 48% more BrdU than control-infected cultures.

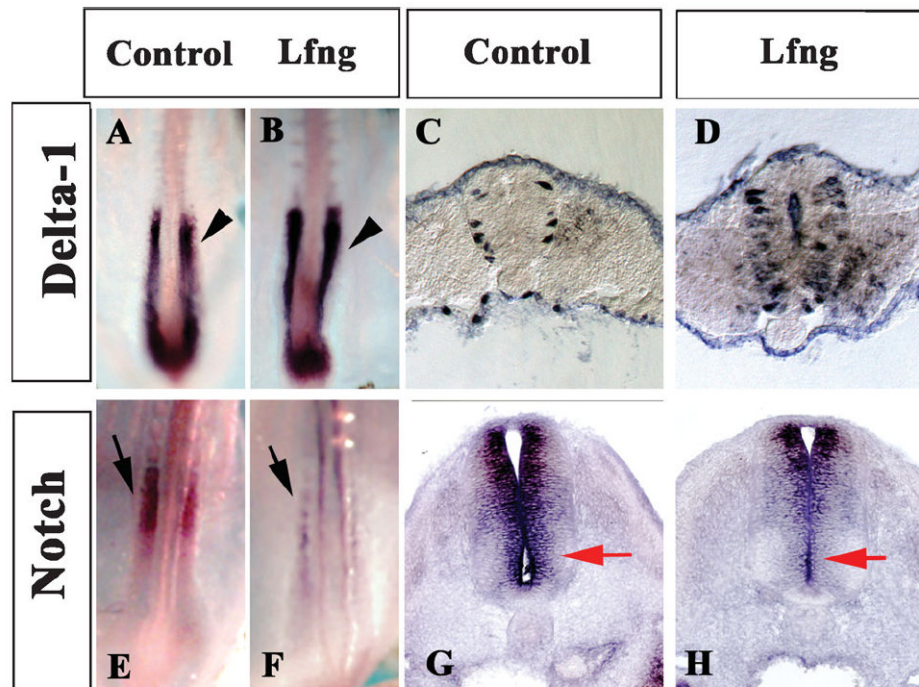


Fig. 6. Lunatic fringe increases Delta-1 and reduces Notch expression

Neural tubes from HH11–13 chicken embryos were infected with either control or lunatic fringe (Lnfg) virus and allowed to develop for 24 hours. Embryos were fixed and processed for *in situ* hybridization for cDelta-1 (A–D) or cNotch-1 (E–H). Notice the increase in Delta-1 in the intermediate neural plate, which gives rise to the neural crest (arrowheads in A,B). More rostral sections through these embryos show more Delta-1-positive cells in the neural tubes of lunatic fringe-infected embryos (C,D). More Delta-1-expressing cells were also observed in the dorsal aorta, these are likely to represent increased numbers of blood cells. The area from which neural crest will originate shows a significant decrease in Notch expression (arrows in E,F). G and H show the distribution of Notch-1 in sections through either control- or lunatic fringe-infected embryos at HH16. The level of Notch appears to be reduced towards the ventricular side of the neural tube after lunatic fringe infection (red arrow).

Table 1

BrdU incorporation after lunatic fringe infection

	Control	Lfng
In vivo ^a	4 ± 2 N = 5	24 ± 6 ^{**} N = 5
In vitro ^b	31 ± 3.6% N = 10	46 ± 4.3% ^{**} N = 10

^{**}
p < 0.005 t-Test

^a Corresponds to the average number of BrdU/p27 positive cells per 20 micron section in the migrating neural crest stream of embryos with uniform retroviral infection.

^b Corresponds to the average percentage of p27 positive cells that incorporated BrdU per neural tube.

For in vivo experiments, chicken neural tubes were infected and incubated for 24hrs after 50mM of BrdU was added close to the heart. For in vitro, infected neural tubes were cultured for 1day allowing neural crest cells to come out, then 10μM of BrdU was added for 30min and cultures were fixed and stained for the p27 viral marker and BrdU labeling.